



# One-pot Synthesis of 4-Aminocyclohexanol Isomers by Combining a Keto Reductase and an Amine Transaminase

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The efficient multifunctionalization by one-pot or cascade catalytic systems has developed as an important research field, but is often challenging due to incompatibilities or cross-reactivities of the catalysts leading to side product formation. Herein we report the stereoselective preparation of *cis*- and *trans*-4-aminocyclohexanol from the potentially bio-based precursor 1,4-cyclohexanedione. We identified regio- and stereoselective enzymes catalyzing reduction and transamination of the diketone, which can be performed in a one-pot sequential or cascade mode. For this, we identified regioselective keto reductases for the selective mono reduction of the diketone to

give 4-hydroxycyclohexanone. The system is modular and by choosing stereocomplementary amine transaminases, both *cis*- and *trans*-4-aminocyclohexanol were synthesized with good to excellent diastereomeric ratios. Furthermore, we identified an amine transaminase that produces *cis*-1,4-cyclohexanediamine with diastereomeric ratios >98:2. These examples highlight that the high selectivity of enzymes enable short and stereoselective cascade multifunctionalizations to generate high-value building blocks from renewable starting materials.

## Introduction

Chiral amino alcohols are widely used as building blocks for many pharmaceuticals such as adrenergic agents<sup>[1]</sup> or drugs against cardiovascular, renal, and inflammatory diseases.<sup>[2]</sup> Additionally, they have also found extensive application as ligands and chiral auxiliaries in asymmetric organic synthesis.<sup>[3]</sup> They are exemplary for many pharmaceuticals: Their syntheses are waste-intensive processes due to the complexity of the target molecules and the necessity to install multiple functional

groups and two or more stereocenters in one building block.<sup>[4]</sup> Therefore, the efficient multifunctionalization of simple starting materials by a cascade of regio- and stereoselective reactions is very attractive, and the development of chemoenzymatic one-pot and tandem reactions has grown as an important research field.<sup>[5]</sup>

In this study we focus on the synthesis of a 1,4-amino alcohol, namely 4-aminocyclohexanol **1**. A modular route giving access to both stereoisomers is desirable: *trans*-**1** is a valuable precursor of drugs such as ambroxol (a secretolytic agent) and lomitubvir (an HCV protease inhibitor),<sup>[6,7]</sup> and also of novel bioactive molecules with a potential clinical interest.<sup>[8]</sup> *Cis*-**1** is required as the building block for substituted isoquinolone derivatives<sup>[9]</sup> and certain spirocyclic compounds.<sup>[10]</sup> Although **1** is a rather simple molecule, no efficient (and highly) stereoselective synthesis of *cis*- or *trans*-**1** has been reported: The hydrogenation of paracetamol by applying mono- and bimetallic catalysts yielded **1** with *cis:trans* ratios of (35–51):(49–65)% of 4-aminocyclohexanol.<sup>[13]</sup> Additionally, the ambroxol isomers containing a *cis*- or *trans*-**1** moiety were prepared starting from cyclohexa-1,3-diene via palladium-catalyzed 1,4-diacetoxylation and allylic substitution with the subsequent hydrogenation of the 4-aminocyclohex-2-enol moieties.<sup>[14]</sup>

Due to their excellent regio- and stereoselectivity, enzymes as catalysts often shorten synthetic routes, as demonstrated in the synthesis of norephedrine, where a two-step enzymatic approach was developed as an alternative to the seven-step classical synthesis.<sup>[15]</sup>

With these precedents in mind, we envisaged two one-pot enzymatic procedures for the stereoselective synthesis of *cis*- and *trans*-4-aminocyclohexanol (Scheme 1) starting from 1,4-cyclohexanedione **2**. This diketone is a very simple, symmetric starting material that can be obtained from succinic acid,<sup>[16]</sup> a

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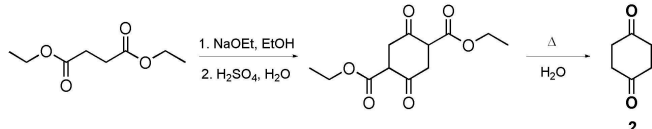
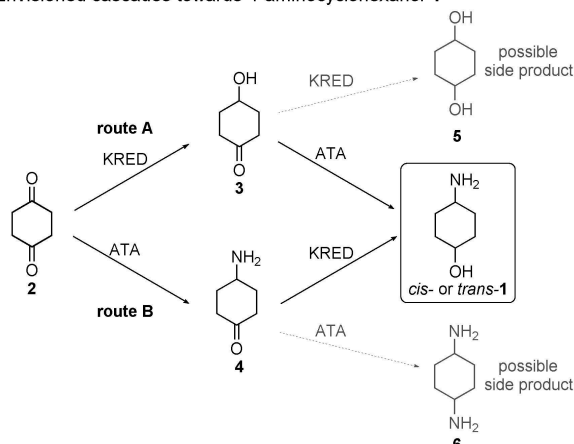
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A) Bio-based route to cyclohexane-1,4-dione **2**B) Envisioned cascades towards 4-aminocyclohexanol **1**

**Scheme 1.** A) Cyclohexane-1,4-dione **2** can be produced from bio-based succinic acid esterified with bioethanol. B) Proposed cascades towards *cis*- and *trans*-4-aminocyclohexanol **1**. The employed enzymes keto reductase (KRED) and amine transaminase (ATA) must be selective to avoid formation of by-products (diol **5** and diamine **6**).

renewable feedstock produced by engineered *E. coli* cells from biomass.<sup>[17]</sup> The first route (route A) would involve the formation of 4-hydroxycyclohexanone **3** from diketone **2** via ketoreductase-catalyzed (KRED) monoreduction coupled with a subsequent transamination by an amine transaminase (ATA). The second option (route B) is to switch the order of steps of the previous sequential approach: a selective monoamination of **2** yields 4-aminocyclohexanone **4**, followed by reduction of the remaining carbonyl group of the intermediate to give **1**. Ideally, these sequences can be run as one-pot or cascade reaction using stereoselective enzymes to obtain **1** in 100% theoretical yield and without isolating any intermediate.

Although this looks straightforward, several issues have to be addressed: The challenge of route A is to find a selective KRED that ideally stops after one reduction step to avoid formation of the unwanted diol side-product **5**. This requires a selective catalyst efficiently discriminating between the very similar di- and hydroxyketones **2** and **3**. To the best of our knowledge, no efficient chemical method for the direct selective monoreduction of **2** is available. For accessing *cis*-1 or *trans*-1 product in the second step, stereoselective ATAs with complementary stereopreferences have to be identified. Ideally, the ATA would act only on the hydroxyketone **3**, but not on the diketone substrate to avoid the formation of **4** and possibly diamine **6** as impurities. In the alternative route B, analogous constraints on substrate- and stereoselectivities of the used catalysts apply: ATA must convert **2**, but not the amino ketone **4**, to prevent formation of by-product **6**; accessing *cis*-1 and *trans*-1 in the next step requires stereoselective KREDs. Addi-

tionally, KREDs should not convert **2** to avoid hydroxyketone or diol side products. Although water is the natural enzyme environment, a number of challenges must be addressed to implement such enzymatic cascades depicted in Scheme 1. For reasons of compatibility, parameters including pH, temperature, substrate concentration, and co-solvents must be optimized, but also specific enzyme activities and stabilities should be balanced and inhibition avoided.

## Results and Discussion

For the first step of route A (Scheme 1), we screened 24 KREDs from the Codexis® KRED Screening Kit and four recombinantly expressed KREDs available in our laboratory collection with a photometric NAD(P)H-assay employing compounds **2–4** as substrates. Half of the enzymes converted **2** and **3** with no or only a slight preference of the diketone **2**. However, there are also notable exceptions with significant selectivity and high activity towards **2** (See Supporting Information). Next, suitable hits with a preference for **2** were employed in biocatalytic reductions of 50 mM of **2** using 2 equivalents of propan-2-ol for cofactor recycling. We used this low excess of the electron-donating propan-2-ol to prevent 'over' reduction to the diol, but still to enable full reduction of one keto-moiety to investigate the regio selectivity of the enzymes. Three KREDs showed excellent activity and selectivity during reduction of **2**, and we observed only small amounts of 1–4% of undesired diol **5** (Table 1, entries 1, 2 and 4). Particularly, KRED from *Lactobacillus*

**Table 1.** Reduction of 1,4-cyclohexanedione **2** employing KREDs.

Entry	Enzyme	Enz. conc. [mg/mL]		
			[ <b>3</b> ] <sup>[d]</sup> [mM]	[ <b>5</b> ] <sup>[d]</sup> [mM]
1 <sup>[a]</sup>	LK-KRED	0.1	45.1	1.2
2 <sup>[b]</sup>	RR-KRED	0.27	44.6	0.8
3 <sup>[c]</sup>	P1-A04	0.55	37.7	5.3
4 <sup>[c]</sup>	P2-C11	0.04	47.2	0.5
5 <sup>[c]</sup>	P2-D11	0.55	36.5	1.4

[a] Reaction conditions: **2** (50 mM), 1.0 mM NAD(P)<sup>+</sup>, propan-2-ol (100 mM, 0.76% v/v), 50 mM sodium phosphate buffer, pH 7.0, 30 °C, 700 rpm, 12 h. *Lactobacillus kefir* (LK)-KRED was produced in *E. coli* and applied as crude cell lysate, [b] Reaction conditions: the same as in [a], except pH 8.0. *Rhodococcus ruber* (RR)-KRED was produced in *E. coli* and applied as crude cell lysate, [c] Reaction conditions: the same as in [a], except 125 mM potassium phosphate buffer, 1.25 mM magnesium sulfate. Enzymes were used as supplied in the Codexis® KRED screening kit, [d] **3** (product) and **5** (by-product) concentrations were determined by GC.

*kefir* (LK-KRED) and KRED-P2-C11 (Codexis) emerged as the best candidates leading to the highest conversions (> 93%) and the

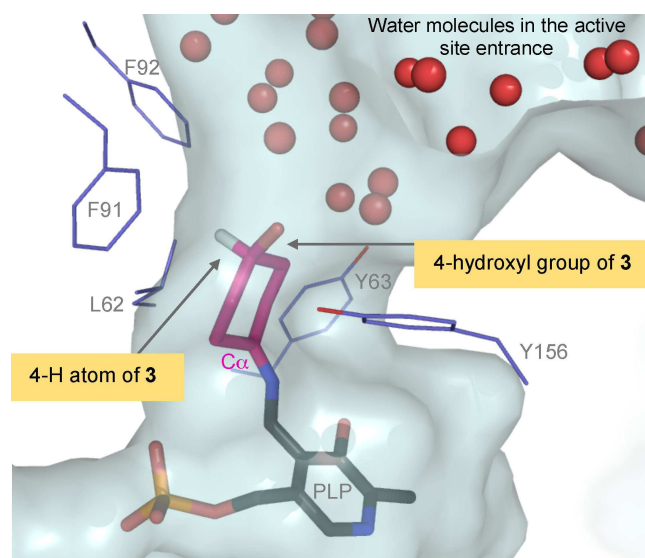
best ratio between the target **3** and by-product **5** (entries 1 and 4).

To optimize the reduction towards high conversions, we varied the percentage of propan-2-ol ranging from 0.76 % to 40 % (v/v). Interestingly, the best performances remained those achieved with the lowest percentage of propan-2-ol as reductant. Thus, although the ratio **3**:**5** increased slightly for LK-KRED and remarkably for KRED-P2-C11 to a higher percentage of the monoalcohol, the conversion gradually declined with higher percentages of propan-2-ol (Table S7, Supporting Information). It is worth highlighting not only the selectivity, but also the high degree of conversion that is reached in the reduction of **2** with the low propan-2-ol content of 2 equivalents, contrasting with previously reported enzymatic keto reductions of other substrates.<sup>[18]</sup> This makes this reaction more attractive from an economic point of view. Compared to previous attempts, this is the first efficient enzymatic access to produce hydroxyketone **3**,<sup>[19]</sup> which itself represents a valuable building block for syntheses of compounds against immune disorders,<sup>[20]</sup> viral<sup>[21]</sup> and neurological<sup>[22]</sup> diseases. Compared to our successful monoreduction of **2** (route A), no KRED was identified having activity towards **4** which prompted us to discard route B (Scheme 1). Alternatively, we were also able to synthesize **3** from the diol **5** by monooxidation employing laccases or following a recently reported methodology for the oxidation of sterically hindered alcohols<sup>[23]</sup> with NaOCl and AZADO, but substrate conversions were below 50 % (see the SI for full details).

After optimizing the first step of the cascade using the KREDs, we screened our collection of 82 recombinant ATAs (wild type ATAs and variants) towards amination of **3** using isopropylamine or alanine as amino donors. For the pre-screening we produced ATAs in microtiter plates and analyzed reactions by TLC with NBD-Cl derivatization to increase sensitivity.<sup>[24]</sup> Among wild-type enzymes, only the (*S*)-selective ATAs from *Ruegeria pomeroyi* (ATA-3HMU), *Ruegeria* sp. (ATA-3FCR), and *Chromobacterium violaceum* (ATA-Cvi) were able to aminate **3** (Table S1, SI). From our variant collection, 9 out of 28 variants of ATA-3FCR and ATA-3HMU converted **3** (Table S2, SI) and several variants from the ATA *Vibrio fluvialis* (ATA-Vfi), despite the inactivity of the wild type (Table S1, SI).

Next, the most promising ATAs were purified (to reduce the possibility of any unspecific reactions of cell lysate enzymes with **3**) and employed in biocatalytic reactions to analyze conversion and stereoselectivity. Most of these ATAs afforded **1** with conversions higher than 60 % using isopropylamine (Table S4, SI). Interestingly, all of them exhibited marked *cis*-selectivity. In particular, the variant ATA-3HMU W63Y led to 92 % of the *cis*-isomer of **1** (Table S4, entry 12, SI), and a variant of ATA-3FCR designed for accepting bulky substrates (ATA-3FCR-4M)<sup>[25]</sup> containing four amino acid substitutions afforded **1** with high (>99 %) *cis*-selectivity. Regarding reactions with D,L-alanine as amino donor, four of the previous enzymes were active but led to poor levels of **1** despite significant consumption of the substrate (Table S5, SI). This substrate depletion also occurred in the absence of an ATA, and the control reactions revealed that the glucose dehydrogenase (used for cofactor recycling) partially catalyzes reduction of **3** to the diol **5**.

Alternatively, we assayed the panel of engineered ATAs from Codexis® with the aim of finding a *trans*-selective enzyme. Most of the ATAs turned out to be very active and catalyzed the amination of **3** with isopropylamine with high conversion, again predom-



**Figure 1.** Modelled quinoid intermediate in ATA-3HMU W63Y formed after the condensation of **3** (magenta sticks) and PMP (black sticks). Subsequent catalytic steps will lead to *cis*-**1**. Note that for obtaining the *trans*-isomer, **3** must bind in an orientation where its 4-hydroxyl group and 4-H atom swap their place. This would hinder solvation of the 4-hydroxyl group. The surface shows the active site entrance tunnel, red spheres are oxygen atoms of water molecules (added by the YASARA program prior to energy minimization). For a discussion of the other conformations please see the Supporting Information and Figure S8.

inantly forming the *cis*-isomer of **1** (Table S6, SI). For example, the ATAs 200, 237, 251 and 254 led to *cis*-**1** with perfect stereoselectivity and conversions of >95 % (Table S6, entries 1, 2, 4, 5, SI). On the other hand, only three enzymes, namely ATAs 113, 234 and 415, exhibited slight *trans*-selectivity (Table S6, entries 10, 17, 20, SI), yielding **1** with *cis*:*trans* ratios of up to 3:7.

Intrigued by our observation that most transaminases showed *cis*-selectivity, we conducted molecular modeling to understand the molecular reasons for this trend. During catalysis, **3** can be bound to pyridoxamine-5'-phosphate in two different orientations that will lead to the *cis*- or *trans*-configuration upon transamination. We modeled the quinoid intermediate leading to *cis*-**1** (Figure 1) in the active site of the ATA-3HMU W63Y variant. The 4-hydroxyl group of the bound **3** (modeled in both chair conformations) can be easily solvated because it faces the solvent molecules that fill the active site entrance tunnel. For *trans*-selective transamination, **3** must be bound in a way orienting its 4-hydroxyl group towards the wall of the active site tunnel (it would take the place of the hydrogen atom shown in Figure 1). Then it would face the hydrophobic amino acids L62 and F91, what impedes its solvation and thus is energetically less favorable. Because these two residues are highly conserved (as revealed from a multiple sequence alignment containing >1000 proteins with sequence identities of 50–99 % with ATA-3HMU), this explains the general *cis*-selectivity of wild-type PLP-fold type I ATA. Unfortunately, the amino acid sequences of the *trans*-selective ATA are not known, what prevented the construction of a model for comparison.

After the initial ATA-screening results, we aimed to increase *trans*-selectivity by optimizing reaction conditions (pH, temperature, DMSO concentration) for the two best ATA candidates 113 and 234. Although the selectivity towards *trans*-**1** formation increased significantly for ATA 113 at higher pH values (Table S10, entries 1 and 3, SI), activities diminished markedly. On the contrary, ATA-234

was able to keep high conversions to the product at the same level as during the screening phase, and an improved selectivity for *trans*-1 (*cis*/*trans* 20:80). We decided to select ATA-234 for further preparative scale experiments, as it also showed higher stability and activity under the investigated conditions.

Once both catalytic steps were reliably established, we explored their combination in a one-pot sequential approach. After the reduction of 80 mg **2** by LK-KRED was completed after 24–48 h (see SI for full details), we added the transamination reaction components containing ATA and co-substrates and adjusted the buffer to the previously identified pH optimum.

Pleasantly, the impact of the KRED and NADPH on the bioamination step was negligible and the target amino alcohols *cis*- or *trans*-1 were obtained with  $\approx 90\%$  conversion (Table 2, entries 1–3). The

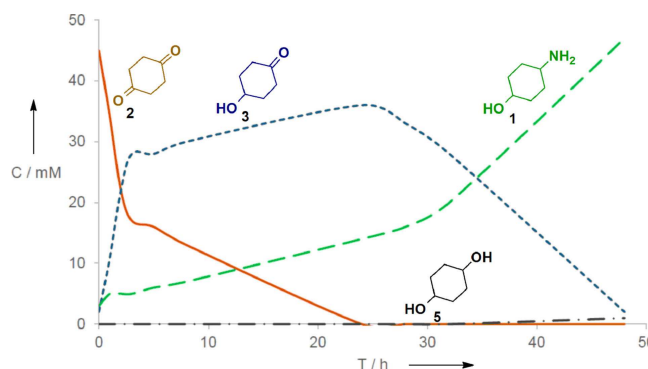


Figure 2. Cascade synthesis of *cis*-4-aminocyclohexanol employing LK-KRED and ATA-3FCR-4M.

Table 2. Synthesis of <i>cis</i> - and <i>trans</i> -1 using one-pot sequential and cascade reactions.							
Entry	Reaction mode	ATA	C <sup>[b]</sup> [%]	1 <i>cis:trans</i> <sup>[a]</sup>	5 [%]	6 [%]	Scale [mg]
1	sequential	3FCR-4 M <sup>[d]</sup>	91	80:20	9	0	80
2	sequential	ATA-200 <sup>[f]</sup>	85	99:1	9	6	80
3	sequential	ATA-234 <sup>[f]</sup>	88	20:80	4	0	80
4	cascade	3FCR-4 M <sup>[e]</sup>	98	80:20	2	0	160
5	cascade	ATA-234 <sup>[f]</sup>	89	25:75	3	0	160
6	cascade	ATA-200 <sup>[f]</sup>	44	99:1	0	48	160
7	ATA only	ATA-200 <sup>[f]</sup>	0	98:2 <sup>[c]</sup>	0	99	80

[a] Substrates and products, *cis:trans* ratios were determined with HPLC and GC, [b] Percentage of total product (*cis:trans*)-1 formed from **3** as detected by HPLC/GC, [c] *Cis:trans* ratio was evaluated by <sup>1</sup>H-NMR (see SI), [d] ATA-3FCR-4 M was produced in *E. coli* and applied as purified enzyme, [e] ATA-3FCR-4 M was produced in *E. coli* and applied as cell lysate, [f] Commercial ATAs were applied as lyophilized enzymes.

reaction mixtures contained small amounts (<9%) of the diol by-product **5** and the ATAs maintained good to excellent stereoselectivities under the one-pot conditions, although ATA-3FCR-4 M performed slightly better in terms of selectivity when used in an isolated reaction. Since aminoalcohol **1** is a very hydrophilic compound, several work-up strategies were tested for its isolation from the aqueous medium. Commonly, derivatization with (Boc)<sub>2</sub>O helps to increase hydrophobicity but due to high isopropylamine concentrations would require using a large excess of the reagent. Solid-phase extraction with Oasis WCX (weak cation exchanger) or Sep-Pak® tC18 Silica columns was not working because **1** started to elute from the columns already during the washing step. The precipitation of **1** with organic acids, such as 3,3-diphenylpropionic acid was also possible,<sup>[26]</sup> but required more optimization steps due to high solubility of the salt. Thus, the best isolation approach was an optimized extraction protocol and crystallization as hydrochloride (see SI), facilitating  $\approx 75\%$  isolated yield in most cases.<sup>[27]</sup> Product purification by column chromatography worked best after Boc derivatization (see Supporting Information).

Importantly, the one-pot reactions were also successful if carried out as cascade reaction. By balancing the used enzyme amounts, diol formation could be further suppressed to 0–3% (entries 4–6). As the tandem reactions required using one common pH for both steps, stereoselectivities decreased for TA-3FCR-4M and ATA-234, but not when ATA-200 was used. A time-course analysis (Figure 2) of the reactions revealed the following challenges of the cascade mode: As expected, the activity of the LK-KRED for the reduction decreased after the

first 20 h of the reaction, when 90% of the diketone **2** was converted. At the same time, the amine transaminase activity was very low: in the beginning, the hydroxy ketone **3** accumulated and its conversion was less than 20% when 90% of the diketone was consumed. However, the transamination rate started to increase when the concentration of the diketone **2** was below 10 mM, indicating that **2** acted as an inhibitor of the transaminase.

Notably, the highest stereoisomeric ratio of *cis*-1 of 99:1 was obtained with ATA-200 (entry 6). Here, we did not observe transaminase inhibition by the diketone **2**, but the ATA also formed 1,4-cyclohexanediamine **6** as by-product, as identified by NMR. Therefore, ATA-200 gives superior results in the sequential mode. Reaction and protein engineering might help to increase regioselectivity for this enzyme. In summary, our approach is the first preparation of 1,4-amino alcohols by a two-enzyme one-pot reaction and therefore extends the literature-known synthesis methods for obtaining 1,2-<sup>[11]</sup> and 1,3-amino alcohols.<sup>[12]</sup>

Due to its broader substrate specificity, ATA-200 can be applied for the synthesis of the diamine **6**: Starting from 80 mg of **2**, transamination furnished the diamine with nearly quantitative conversion (entry 7). Remarkably, <sup>1</sup>H-NMR revealed that the *cis* isomer was formed with excellent *cis:trans* ratio of 98:2 (Table 2, Entry 7, see Figure S23 for NMR spectra). *Cis*-**6** is used as building block for tyrosine kinase inhibitor synthesis<sup>[28]</sup> and as ligand (1,4-dach) in the platinum complex kiteplatin [Pt (1,4-dach)Cl<sub>2</sub>], which recently has been shown to be active against colon cancer cells lines that are resistant to cisplatin and oxaliplatin.<sup>[29]</sup> Furthermore, *cis*- and *trans*-**6** are used to modify the properties of polymers such as polyamide.<sup>[30]</sup> To the best of our knowledge, the preparation of *cis*-**6** by transamination represents its first highly stereoselective synthesis. The potential of ATA200 for synthesizing chiral diamines is remarkable and will be explored in the future, as only few ATAs are known that can form this class of compounds. One example is a variant of the *Paracoccus denitrificans* transaminase that converts the cyclic isosorbide diketone to give the diamine with low yields.<sup>[31]</sup>



## Conclusions

In conclusion, we developed a totally enzymatic one-pot synthesis of *cis*-1 from a potentially renewable bio-based substrate. The molecular architecture of the active site of PLP fold type I ATA causes their general *cis*-selectivity. We identified only three trans-selective ATA variants, which also facilitated the preparation of *trans*-1, although with lower stereo selectivity. Our approach is an attractive alternative to the established 3–5-step preparation of *rac*-1, starting from phenol or nitrobenzene (See Scheme S2 in the Supporting Information). The approach can be operated in sequential or in tandem mode and represents an efficient functionalization of a simple starting material by a sequence of precisely arranged reactions. It is a nice example how cross-reactivity — a common challenge in cascades — can be avoided by identifying two regioselective enzymes that discriminate between two very similar substrates such as the employed diketone and hydroxyketone **2** and **3**. The developed synthesis avoids intermediate product isolation, metal catalysis, minimizes by-product formation and affords high reaction yields.

## Experimental Section

All experimental details on analytics, enzyme production and screening, and all biocatalysis reactions are given in the Supporting Information. Below follows a representative procedure for the concurrent cascade synthesis of *cis*-4-aminocyclohexanol **1**: The reaction was performed at a 1.5 mmol scale (concentration of 1,4-cyclohexanedione was 50 mM) in an opened one-necked flask equipped with a magnetic stirring bar (stirring at 250 rpm) and a thermometer (at 30 °C). Freshly isolated cell lysate of LK-KRED (0.2 mg/mL) and ATA-200 (2 mg/mL) were added to 30 mL of 100 mM potassium phosphate buffer, pH 7.5, containing 1 mM NADP<sup>+</sup>, 0.76 % (v/v) isopropanol (100 mM), 1 mM MgCl<sub>2</sub>, 500 mM isopropylamine, 1 mM PLP, and 2 % v/v DMSO. After 48 hours, the reaction was stopped after 44% conversion (Table 2) and the product was isolated as described in the Supporting Information and afforded light yellow crystals of *cis*-4-aminocyclohexanol hydrochloride (99:1 % *cis:trans* ratio).

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## Conflict of Interest

The authors declare no conflict of interest.

**Keywords:** amino alcohols • asymmetric catalysis • enzyme catalysis • amine transaminase • ketoreductase

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